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Soil protistology rebooted: 30 fundamental questions to start with

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Abstract

Protists are the most diverse eukaryotes. These microbes are keystone organisms of soil ecosystems and regulate essential processes of soil fertility such as nutrient cycling and plant growth. Despite this, protists have received little scientific attention, especially compared to bacteria, fungi and nematodes in soil studies. Recent methodological advances, particularly in molecular biology techniques, have made the study of soil protists more accessible, and have created a resurgence of interest in soil protistology. This ongoing revolution now enables comprehensive investigations of the structure and functioning of soil protist communities, paving the way to a new era in soil biology. Instead of providing an exhaustive review, we provide a synthesis of research gaps that should be prioritized in future studies of soil protistology to guide this rapidly developing research area. Based on a synthesis of expert opinion we propose 30 key questions covering a broad range of topics including evolution, phylogenetics, functional ecology, macroecology, paleoecology, and methodologies. These questions highlight a diversity of topics that will establish soil protistology as a hub discipline connecting different fundamental and applied fields such as ecology, biogeography, evolution, plant-microbe interactions, agronomy, and conservation biology. We are convinced that soil protistology has the potential to be one of the most exciting frontiers in biology.

1. Introduction

Protists are everywhere, in aquatic and terrestrial ecosystems, free-living, and as symbionts (including parasites) of many organisms including humans. These usually single-celled or colonial microorganisms are by far the most diverse eukaryotes (Adl et al., 2012) and their species-numbers might easily exceed 10 million (Global Soil Biodiversity Atlas; www.globalsoilbiodiversity.org). Since the term ‘protista’ was introduced (Haeckel, 1866), profound taxonomic re-orderings have taken place. The vast majority of eukaryotic lineages has been shown to be protists, with the exception of the derived monophyletic multicellular lineages: animals, plants, and some fungi (Burki, 2014).

Electron microscopy and molecular phylogenies have revealed that both algal and protozoan lineages are intermingled throughout the eukaryote phylogenies (Delwiche, 1999; Burki, 2014), and hence it is less confusing to use Haeckel's broader category of 'protist'. Similarly, the classical protozoan morphological categories: flagellates, testate and naked amoebae – but not ciliates - are not monophyletic but distributed across the eukaryotic tree of life (Adl et al., 2012). A snapshot of the immense morphological and phylogenetic diversity of soil protists is visualized in Fig 1. We therefore recommend to use 'protist' as a term for all single celled phototrophic, mixotrophic and heterotrophic eukaryotes, with the exception of fungi.

The huge diversity of protist species has only recently become evident as many morphospecies recognizable under the microscope were shown to hide many cryptic species (Boenigk et al., 2012a). This 'dark matter of biodiversity' suggests that protist taxon richness has been considerably underestimated. A recent study of environmental eukaryotic diversity based on state-of-the-art high-throughput sequencing (HTS) showed that protists are considerably more diverse than plants and animals in the sunlit zone of oceans (de Vargas et al., 2015). HTS studies of soil protists have shown a wide diversity of non-phagotrophic protists and the diversity of protists in soils is at least as diverse as that in aquatic systems (e.g. (Bates et al., 2013; Geisen et al., 2015c). Nevertheless, soil protists are much less well studied than their aquatic counterparts and this gap is increasing (Fig. 2a).

Soil protists have received relatively little attention mainly due to methodological challenges, especially their isolation from the opaque soil matrix. These, however, do not entirely explain why soil protists are relatively less studied than other soil organisms, especially bacteria, fungi and nematodes (Fig. 2b). The volume of work on microbial bacteria and fungi far outweighs protist studies, possibly because of their direct role as primary decomposers, and they represent monophyletic groups that can more easily be studied with various targeted methodological approaches (Foissner, 1987; Mitchell, 2015). Even soil viruses have been subject to more studies than soil protists, despite being extremely challenging to study (Fierer et al., 2007) and their uncertain functional importance in soils. The under-studied nature of soil protists is exemplified by a comparison between research on protists and on soil archaea, a domain erected in 1990 and reported to be functionally important in soil only decade ago (Leininger et al., 2006; Bates et al., 2011). Historically studies mentioning soil protists in the title were eight times more abundant than those including archaea (Fig. 2b, Supplementary Table 2). However, in the last 15 years, this pattern entirely changed; studies on soil protists decreased by 15% while those on other common soil organisms increased by at least 30%, especially soil archaea which increased by 88% (Fig. 2b, Supplementary Table 2).

The relative decline of papers on soil protists strongly contrasts with what we now know about their ubiquity, diversity, and perhaps more importantly, their functional significance. Soil protists can both make an important contribution to primary production (Jassey et al., 2015; Schmidt et al., 2016) and play a key role in the decomposition pathways as consumers of bacteria (Clarholm, 1981; de Ruiter et al., 1995), fungi, other protists, and small invertebrates; they can also act as parasites of plants and animals (Adl and Gupta, 2006; Jassey et al., 2012; Geisen, 2016b). As predators, protists transfer nutrients to higher trophic levels in the soil foodweb (de Ruiter et al., 1995; Crotty et al., 2012). Protist predation also stimulates microbial activity and nutrient cycling via the microbial loop, thus stimulating plant growth (Bonkowski and Clarholm, 2012) and representing an important link between aboveground and belowground components.

The functional significance, abundance, environmental sensitivity, rapid response times and increasing ease of analysis of soil protists also makes them invaluable bioindicators of a variety of aspects of environmental change (Foissner, 1987; Gupta and Yeates, 1997; Payne, 2013). A particular example of this is in paleoecology, where the hard shells of testate amoebae, diatoms and foraminifera are widely used in the reconstruction of past environments and past climate change over a range of timescales (Mitchell et al., 2008; Adl et al., 2011; Charman, 2015).

Most of these applications are, however, based on a few often small-scale studies. Thus significant taxonomic and functional aspects remain largely untouched. Our aim in this report is to pool expert knowledge and opinion across the diverse field of soil protistology and soil microbial ecology to identify major knowledge gaps that need to be addressed and their significance for soil processes and ecosystem services.

2. Materials and Methods

2.1 Approach to identify the 30 most relevant questions

Our aim was to review research gaps both in the field of soil protistology and in general soil biology with a special focus on protists. In line with recent studies (Sutherland et al., 2013; Seddon et al., 2014), we aimed to pool community expertise to identify the most important questions in different broad categories. We modified previously-used methods (Sutherland et al., 2013; Seddon et al., 2014) to obtain a list of most interesting questions through a democratic, transparent, multi-step curation process.

The participants in this process are involved in a wide range of research areas, with self-determined primary research area expressed as being ecology (62%), palaeoecology (12%), evolution (9%), biogeography (6%), phylogeny (6%), taxonomy (3%), parasitology (3%). Each participant formulated up to 10 questions that they believed were most relevant for their future research. The resulting 368 questions were then compiled via an integrative group effort into consensus questions and placed into six major categories following a discussion by 16 of the participants at the German Society for Protozoology meeting in February 2016. We included very broad, general questions as well as highly specialised topics into similar scaled consensus questions.

The resulting consensus questions were then re-evaluated and groupings adjusted in a vote. These questions (Supplementary Table 1) were sent out to all 47 participants, who individually indicated up to 12 priority questions with at least one being allocated in each of the six following categories: (i) Morphology, Phylogeny, Taxonomy, Evolution and Physiology, (ii) Diversity, Community Composition and Biogeography, (iii) Interactions among Protists and other Organisms, (iv) Functions of Protists, (v) Global Change, Bioindicators and Applications, and (vi) Methodology.

All 47 participants were asked to provide their key scientific expertise and literature references for studies that (partly) addressed individual questions. Finally, minor comments raised by individual participants during the vote were integrated to clarify the questions and give consistent formatting without changing the meaning of the questions that had been voted upon.

All individual votes were combined and five questions per category chosen to result in the final list of 30 key questions. When more than one question received the same number of votes (as present in categories 1, 2, and 4), these questions were sent out to all 47 participants for another vote on the selected questions only.

2.2 Potential limitations

Biases in broad-scale studies are impossible to avoid (Sutherland et al., 2013). On the other hand, the more interdisciplinary the panel of authors is in terms of cultural and societal background and specific scientific expertise, the more biases are reduced. Researchers working on soil protists are often ecologists, whereas taxonomists, phylogeneticists, and physiologists more often focus on aquatic taxa that are easier to isolate and cultivate. Indeed, participants who indicated ecology as their first expertise dominated our list of participants (62%). Ecology, however, is a broad field and our division into finer categories such as biogeography, palaeoecology, community structure, and interactions resulted in a broad diversification into different subcategories. Additionally, 21% of the participants indicated topics such as taxonomy, phylogeny, evolution, and physiology as their main expertise corroborating the wide diversity of research fields among the co-authors.

The majority of participants are PhDs (Professor: 38%; Graduated scientists: 29%; Post-doc: 24%) with an average number of publications on protists of 43 (minimum = 1; maximum = 230). A high proportion of the participants work on multiple ecosystems (41%). Many focus on testate amoebae (41%) although 26% of them work on multiple morphogroups (ciliates, heterotrophic flagellates, amoebae, etc.). A majority (74%) of participants have a European background, but Asia, North and South America are also well represented, thus reducing potential impacts of geographic origin. Furthermore, most participants have international collaborations that partly compensate for gaps in the geographic distribution of individuals.

Despite these potential limitations, we found few biases in the way participants replied to questions (Supplementary Results 1). Most participants (70%) selected questions evenly distributed across the six categories, except a small group of people mostly constituted of researchers from the same institute and/or with the same kind of expertise (phylogeny, taxonomy and evolutionary; see Supplementary Results 1). This small group allocated 45% of their votes to the category (ii). Except this small bias, most participants selected questions regardless of their experience, age, geographic background, and most importantly, their expertise and group of interest.

Questions were differently formulated, hence we had to make decisions and remove some nuances as we merged similar questions. This resulted in some discussions about how questions should be best stated and consequently combined and grouped into non-predetermined categories. However, we preferred to receive non-restricted questions to stimulate lateral thinking as previously suggested (Sutherland et al., 2013); due to intensive exchange and to a democratic group effort at all steps of the procedure, we are convinced that we have reached a consensus format.

3. Results and Discussion

3.1 The 30 most relevant consensus questions

From the 107 questions in the final vote, 94% received at least one, 79% two, 67% three and 50% five votes showing that the pre-selected questions had a wide general appeal to the scientific experts involved (Supplementary Table 1). Therefore, all questions seem to be relevant for future studies that focus on soil protists. However, as we aimed at providing a highly specific list of the major research gaps and open challenges in soil protistology, we only provide the top-ranked 30 questions classified in six major categories that most researches voted upon.

3.2 Categories

I Morphology, Phylogeny, Taxonomy, Evolution and Physiology

1. How long can protists survive in an encysted form? What are the tolerances of (encysted) protists to stress and what is the importance of cysts for ecosystem resilience?
2. How much morphological and genetic variability exists within soil protists?
3. How do species that occur in both aquatic and soil systems adapt to differing demands?
4. What are the phylogenetic relations of true soil to aquatic protist taxa and how often have soils been colonized by aquatic protists and vice versa?
5. How widespread is sex in soil protists?

Linking the individual topics of this category is one of the major tasks confronting soil protistology. The coupling of morphology and phylogeny is crucial to obtain a stable taxonomic framework for protists. This is, for instance, crucial to answer evolutionary questions on the origin of eukaryotes (López-García and Moreira, 2015). Soil protists may have an important role to play in such research as most taxa likely remain unknown and novel higher-level taxonomic groups are continuously being discovered (Berney et al., 2015; Bass et al., 2016; Singer et al., 2016; Tice et al., 2016). Soil protists might fill remaining phylogenetic gaps from better-studied aquatic taxa to improve phylogenetic resolution within and between protist clades, as strict soil protist clades seem to be common (Bass et al., 2016). Sequencing whole genomes will reveal ancient traits of eukaryotes and potential changes in their function during the evolution of eukaryotes. In this respect, soil protists must certainly play a key role for understanding the evolution of the eukaryotic cell and, therefore, of life as a whole.

While the morphological and phylogenetic framework for ciliates is reasonably well established (Lynn, 2008; Foissner, 2016), it remains rudimentary for other morphogroups as well as phylogenetic clades of protists (Kosakyan et al., 2016). The taxonomy of the groups has profoundly benefited from (mainly) 18S rRNA gene-based characterisations that have often led to drastic changes in phylogenetic placements of individual species, genera, families or even orders (Boenigk et al., 2012a; Berney et al., 2015; Bass et al., 2016). This is exemplified by the morphologically and functionally diverse Cercozoa, which was the first protist clade inferred solely based on molecular phylogenetic information, and has become home to ever more morphologically different organisms (Bass et al., 2016). Therefore, the true extent of morphological and genetic variability in different groups of soil protists remains largely unknown and is a key missing gap for future studies (Q2).

A key feature of soil protist species is their capacity for cyst formation as this allows them to resist constantly changing conditions, especially with respect to moisture and temperature. Furthermore, given that protists can excyst after decades, even millennia (Shmakova et al., 2016), cyst formation may protect species from becoming extinct at local or even at the global scales, influence population dynamics and maintain biodiversity (Corliss and Esser, 1974; Jones and Lennon, 2010) The importance of the cyst bank in ecosystem functioning and resilience remains largely unknown and have consequently been identified as a key element for future studies (Q1). More generally, this applies to all specific (physiological) adaptations of soil protists in comparison to their aquatic relatives (Q3, Q4) and to reproduction (Q5)..

II Diversity, Community Composition and Biogeography

6. What is the real diversity and community structure of soil protists in different systems (e.g. soils, rhizosphere, (plant) endosphere)?
7. How similar are the diversity patterns of soil protists and other soil biota along ecological gradients, and to what extent do different environmental factors shape their respective diversity?
8. What abiotic environmental factors influence the distribution and community composition of protists, and how?
9. How cosmopolitan are protists and how many endemic soil protist species are there?
10. What are dominant groups of soil protists in terms of turnover, abundance and biomass?

We are progressively shedding light into the soil 'black box'; however, knowledge on protists lags behind that of other groups (Fig. 2) (Wilkinson, 2008). Traditional studies have focused exclusively on a few of the 'classic' morphogroups, especially ciliates and testate amoebae, at least partly due to their ease of isolation and feature-rich morphologies (Foissner, 1999). Despite dominating many soil protist communities in terms of numbers and diversity, flagellates and naked amoebae have remained understudied, due to their often smaller sizes, lack of diagnostic features when studied by light microscopy, and the need to establish specialised enrichment cultivation for their isolation from soils (Berthold and Palzenberger, 1995; Foissner, 1999; Smirnov and Brown, 2004; Tikhonenkov et al., 2010). The development of molecular tools such as DNA barcoding and metabarcoding has considerably improved the situation in the last decade (Pawlowski et al., 2012) and allowed a phylogenetically based (morphogroup-independent) and consequently much more detailed analysis of the entirety of soil protist communities. These studies have revealed an enormous diversity of protists inhabiting soils, a phylogenetic diversity that might be similar to that of bacteria (e.g., (Bates et al., 2013; Geisen et al., 2015c; Mahé et al., 2017). Also, groups of protists previously almost unknown from soils have been shown to be common e.g. choanoflagellates, foraminifera, dinoflagellates, parasitic apicomplexans and pathogenic oomycetes (Bates et al., 2013; Geisen et al., 2015c; Grossmann et al., 2016; Mahé et al., 2017). Therefore, we are only beginning to understand the diversity of soil protists (Q10), which part is active, how this diversity differs in different soil environments (Q6), how protist communities are structured by, e.g., abiotic factors (Q7, Q8) (Geisen et al., 2014a; Lentendu et al., 2014; Geisen et al., 2015c; Dupont et al., 2016).

In addition, the biogeography of (soil) protists has been addressed in some studies, but it is still unclear which and how many groups display a restricted biogeography and what the factors are that shape these distributions (Q8, Q9). Although distribution of bacteria may support the hypothesis that "everything is everywhere, but, the environment selects" (Baas-Becking, 1934), its extrapolation to protists has been countered, particularly, by work on ciliates and testate amoebae (Foissner, 2006, 2008; Fernandez et al., 2016). The diversity and biogeographical distribution of protists, is, therefore, one of degree (rather than all cosmopolitan or all limited) and the possibility exists that the investigation of cryptic diversity within morphospecies will allow a finer-scale resolution of these questions.

III Interactions among Protists and other Organisms

11 How do protist taxa affect the composition of the soil microbiome and what other important interactions take place?

12 What are the biotic interactions of soil protists with other taxonomic groups, and how are protists linked within the soil food web?

13 What is the relative contribution of nutrient cycling (i.e. the microbial loop) versus modification of the rhizosphere microbiome in protist-induced stimulation of plant growth?

14 What are the mechanisms by which individual soil protist species affect plant performance, and do those mechanisms differ between plant species?

15 What is the impact of protists on the community functioning of other soil microbes?

Soil protists are still predominantly considered as being mainly bacterivorous (Bradford, 2016; Geisen, 2016a). Differential feeding by protists stimulated by bacterial volatiles modifies the community composition of bacteria (Bonkowski, 2004; Glücksman et al., 2010; Schulz-Bohm et al., 2017), which results in functional changes in the bacterial community structure (see next section). Many free-living bacteria can, in turn, defend themselves against certain protist predators and even kill them (Greub and Raoult, 2004; Jousset et al., 2006). Several bacteria, viruses, and even other protists can also parasitize protist hosts (Barker and Brown, 1994; Raoult and Boyer, 2010).

The prey spectrum of protists has, however, repeatedly been shown to be much more diverse than bacteria. Indeed, archaea (Ballen-Segura et al., 2017), fungi (Gupta and Germida, 1988; Ekelund, 1998; Adl and Gupta, 2006; Geisen et al., 2016), other protists (Page, 1977; Jassey et al., 2012), and nematodes (Bjørnlund and Rønn, 2008; Geisen et al., 2015b) constitute prey for diverse protist species. Recently, HTS approaches have revealed the ubiquitous presence and dominant roles of protist parasites and pathogens in soils, and they likely represent a key component controlling other soil organisms including larger soil metazoans (animals) and plants (Geisen et al., 2015a; Dupont et al., 2016; Geisen, 2016b). This draws attention to the enormous complexity and importance of protist interactions with other organisms (Bonkowski, 2004).

Due to our limited knowledge of protist diversity and because most studies have used only one or few protists as models, we lack understanding about most aspects of how soil protist communities interact with other organisms. Disentangling the diverse interactions of protists with other soil organisms (Q11, Q12, Q15), the exact mechanisms (Q14) and the resulting importance for functioning (Q13, Q14), therefore, are key knowledge gaps necessitating future research.

IV Functions of Protists

16 What is the importance of soil protists in biogeochemical cycling?

17 How much functional redundancy is there in the soil protist community?

18 Does increased protist diversity affect ecosystem functioning?

19. What is the comparative importance of eukaryotic microbes vs. prokaryotes in driving key soil processes?
20. Which individual functions are performed by distinct groups, and what is the entire functional diversity of soil protists?

Many acknowledged functions of soil protists are attributed to interactions with other organisms as outlined above. Especially important is the role of protists in driving the microbial loop, i.e. releasing nutrients (particularly nitrogen) bound in bacterial prey. The microbial loop has been demonstrated both in aquatic (Azam et al., 1983) and soil systems (Clarholm, 1985). This ground-breaking research identified protists as important drivers of the global ecosystem. Subsequent work on the microbial loop demonstrated that differential feeding by protists on bacterial prey is beneficial for plant growth (Bonkowski, 2004; Rosenberg et al., 2009). The main focus in earlier studies was, however, mainly on nitrogen cycling, and the importance of protists for cycling of other elements such as carbon and phosphorus has been relatively neglected, with few exceptions (Cole et al., 1977; Gupta and Germida, 1988; Treonis and Lussenhop, 1997; Frey et al., 2001; Murase et al., 2011; Eisenhauer et al., 2012; Jassey et al., 2015). Protists might even play a role in silica cycling as some use Si as reinforcing elements or in an exoskeleton (Aoki et al., 2007; Creevy et al., 2016). More thorough investigations about the functional roles of additional protist species and communities as a whole will likely reveal insights into the importance of protists in biogeochemical nutrient cycling. This was identified by most participants of this study as *the* most important question for future research (Q16).

In contrast to free-living protists, plant pathogenic protists, such as oomycetes or plasmodiophorids, have, not surprisingly, attracted considerable attention due to their agro-economic impact (Anderson et al., 2004; Bell et al., 2006; Neuhauser et al., 2014). These were, however, until very recently often considered as ‘fungi’ (Schardl and Craven, 2003; Gams et al., 2011). Similarly, soil protists with immediate relevance for human diseases such as those directly harmful to humans (Schuster, 2002; Siddiqui and Ahmed Khan, 2012; Geisen et al., 2014b) and those that act as “Trojan horses” harbouring human-pathogenic bacteria (Brown and Barker, 1999; Molmeret et al., 2005) have received considerable attention. In turn, the role of protists in plant disease control due to, e.g., increasing bacterial biocontrol agents (Jousset, 2012) or by directly feeding on plant pathogens (Old and Chakraborty, 1986; Geisen et al., 2016) has received comparatively little attention. In line with their importance in nutrient cycling and as biocontrol agents, the role of individual protist species and that of protist diversity for the general functioning of soils and ecosystems (Q17, Q18, Q20), also in comparison to other groups of microbes (Q19), were identified as important questions to be addressed in future studies.

V Global Change, Bioindicators and Applications

21. How do changing climatic patterns affect the diversity of, community structure of and ecosystem services provided by soil protists?
22. Which protist clades can be used as bioindicators to assess soil properties, ecosystem state, and anthropogenic impacts? How could this be implemented?
23. Why are some species more sensitive to environmental change than others, why do some respond faster to environmental factors?
24. How can protists be used for nutrient mobilization and biocontrol in cropping systems?
25. What is the importance of soil protists for biodiversity conservation and ecosystem management and restoration? Should we protect particular species or habitats?

Protist communities are often studied as bioindicators of past and present climatic conditions, land use changes and pollution (Gupta and Yeates, 1997; Mitchell et al., 2008). Abiotic changes affect protists in species-specific ways, thus forming the basis for their use as bioindicators (Fournier et al., 2012). They may, for instance, provide information on soil state in agro-ecosystems (Foissner, 1997, 1999; Bharti et al., 2015). Testate amoebae and their subfossil remains have been used to evaluate wetland hydrological conditions, applied, for instance, in studies of peatland restoration (Marcisz et al., 2014) and reconstruction of Holocene environmental change (Turner et al., 2014; Lamentowicz et al., 2015; Payne et al., 2015). However, more generally, there has been little progress on evaluating protists as bioindicators even though reliable indicators to assess soil quality continue to be of high relevance (Griffiths et al., 2016) as also revealed here (Q22). Application of protists for stimulating plant performance in terms of nutrition, growth, productivity and disease suppression holds great promise but has received little attention (Q24)

Effects of ongoing global climate change and human impact on the environment are the focus of increasing scientific attention. Global warming has been shown to alter the abundance and community structure of protists (Tsyganov et al., 2011; Jassey et al., 2013) in the limited number of studies that have been done. Predicted changes in precipitation regime will likely affect water availability, which will impact protist communities directly (Clarholm, 1981; Bates et al., 2013; Geisen et al., 2014a). Elevated atmospheric CO_2 has also been shown to increase abundance and changes community structure of rhizosphere protists, possibly due to increased plant productivity and enhanced release of root organic exudates (e.g., (Treonis and Lussenhop, 1997; Anderson and Griffin, 2001; Rønn et al., 2002)). Increased air pollution by nitrogen, sulphur, tropospheric ozone and metals are also likely to alter protist abundance and diversity (Meyer et al., 2012; Payne et al., 2012; Payne et al., 2013). Most of these studies focused on testate amoebae, but it is important to study how global environmental changes affect entire protist communities (Q21, Q23, Q25) as these changes are likely to have significant impacts on ecosystem functioning/services and, consequently, on human welfare, and may provide more informative markers of environmental change.

VI Methodology

26 What is the most practical taxonomic unit to measure protist diversity?

27 How can we standardize and calibrate cultivation based and molecular methods to reliably quantify soil protist abundance, diversity and activity?

28 How should sampling be performed to adequately evaluate soil protist diversity?

29 At what scales (temporal, spatial/physical, morphological, phylogenetic) should we study protists to fully understand their diversity and function in soil; which one should be prioritized?

30 How can we infer functional traits of soil protists based on morphology or phylogenetic affiliation, and what taxonomic resolution is needed?

Diverse methods are used to study community structures of soil protists. Even with respect to more classical culturing and morphological observational techniques, the application of methods of non-protistological disciplines, such as mycology, have the potential of broadening our perspectives on the soil protist community (Spiegel et al., 2004). However, especially recent developments in molecular sequencing technologies, have changed and will continue to change our knowledge about protist diversity and community structure in soils (Bates et al., 2013; Geisen et al., 2015c). However, some issues relating to HTS-based efforts remain as they provide relative abundances of taxa without providing information on absolute abundances. For example PCR-based HTS efforts have been shown to artificially alter the observed community structure of soil protists, a problem which needs to be solved to decipher their real community structure (Geisen et al., 2015a). PCR-free 'omics-approaches', i.e. metagenomics and metatranscriptomics, might resolve some of these issues (Geisen et al., 2015c; Jacquiod et al., 2016). Indeed, these sequence-based omics approaches and sequence-independent metaproteomics provide valuable information not only on taxonomic diversity but also on their potential functions (Prosser, 2015). Calibrating, standardizing and adopting community-defined methodologies to study soil protists will, consequently, be key for cross-study comparisons (Q27) and correct sampling and analyses through different scales need to be defined *a priory* (Q28, Q29). Furthermore, it is essential to identify the most meaningful taxonomic levels to use in the study of diversity and functioning of soil protists (Q26), but even the definition of a species remains a challenge (Boenigk et al., 2012b) and integrating morphology to phylogeny to function remains missing (Q30).

In addition, medical and novel imaging techniques applied to soil are revolutionising *in situ* work allowing us to study protist species in undisturbed soil and on plant roots. These include applications of NanoSIMS technology to precisely locate isotopic markers and isotopic composition of material in fixed preparations and to study dynamics of nutrient fluxes (Stockdale et al., 2009), which allows tracing nutrient flow from microbial prey to protist predator and further in the food web in high resolution. This will allow detailed investigations how protists selective interact in microsites with their prey, how nutrients become released and where they are translocated. Applications of a variety of X-ray based synchrotron spectroscopy and tomography with undisturbed soil is becoming technically feasible and permits the study of dynamics and fluxes at a very fine resolution without interfering with the matrix (Keyes et al., 2013). The ability to use soils with intact fine roots, and examining undisturbed natural soil communities finally provides access to rhizosphere processes. Techniques to measure and analyse chemically soil community molecular interactions and communications are now only a few steps away.

3.3 (Partial) knowledge gaps and future directions

In this paper we provide a guide to 30 highly relevant questions for future studies in soil protistology. Research has already been conducted on many of these questions. Literature searches and personal knowledge of the literature allowed us to identify studies that addressed 91 % of the initial and 97 % of the final questions. However, many of these studies focus on organisms other than soil protists (e.g. aquatic protists or non-protist microbes), and may not be directly applicable to the situation with soil protists. The fact that these 30 questions have been identified by our pool of experts strongly implies that previous research has been insufficient to provide conclusive answers. In Supplementary Table 1 we provide an extensive bibliography of previous research relevant to addressing these questions. This bibliography will be a valuable literature guide to the current state of the art on soil protistology.

We are beginning to understand many aspects of soil protist biology, as we are identifying the hyperdiverse nature of protist communities, determining their (a)biotic drivers, deciphering interactions with other organisms, and shedding light on their importance in ecosystem dynamics. So far, however, we are only seeing the tip of the iceberg. Addressing many of the 30 questions highlighted here will undoubtedly reveal novel insights, not only into soil protists, but also into other organisms, soils, and fundamental ecological processes. We hope that these questions will be used to catalyse soil protistology and to build research agendas for the future. More specifically, we encourage both protistologists and researchers in closely related fields to consider these questions carefully and to use them to develop new and innovative individual and collaborative projects. With newly available techniques, an increase in knowledge and a growing awareness of the importance of soil protists, we are at the start of a bright future for soil protist research!

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Author contributions

SG, EADM and EL designed the study; all authors sent initial questions. SG, TH, VEJJ, DJGL, DS and DW compiled the information and analysed data for Fig. 2. JW, MB, AF, EL, VK, ALT, SK, FS, AS, FE, SG provided photographs that DS, SG, EL and EADM compiled in Fig. 1. SG, EL, EADM, MB, VK, KM, DJGL, MWB, MM, DMW, BSG, SA, RR, AMFD integrated, modified and established the final questions being sent out to all participants. All authors voted on the final list of questions and provided references for separate questions. SG and EL wrote the first draft of the manuscript and all authors contributed substantially to revisions.

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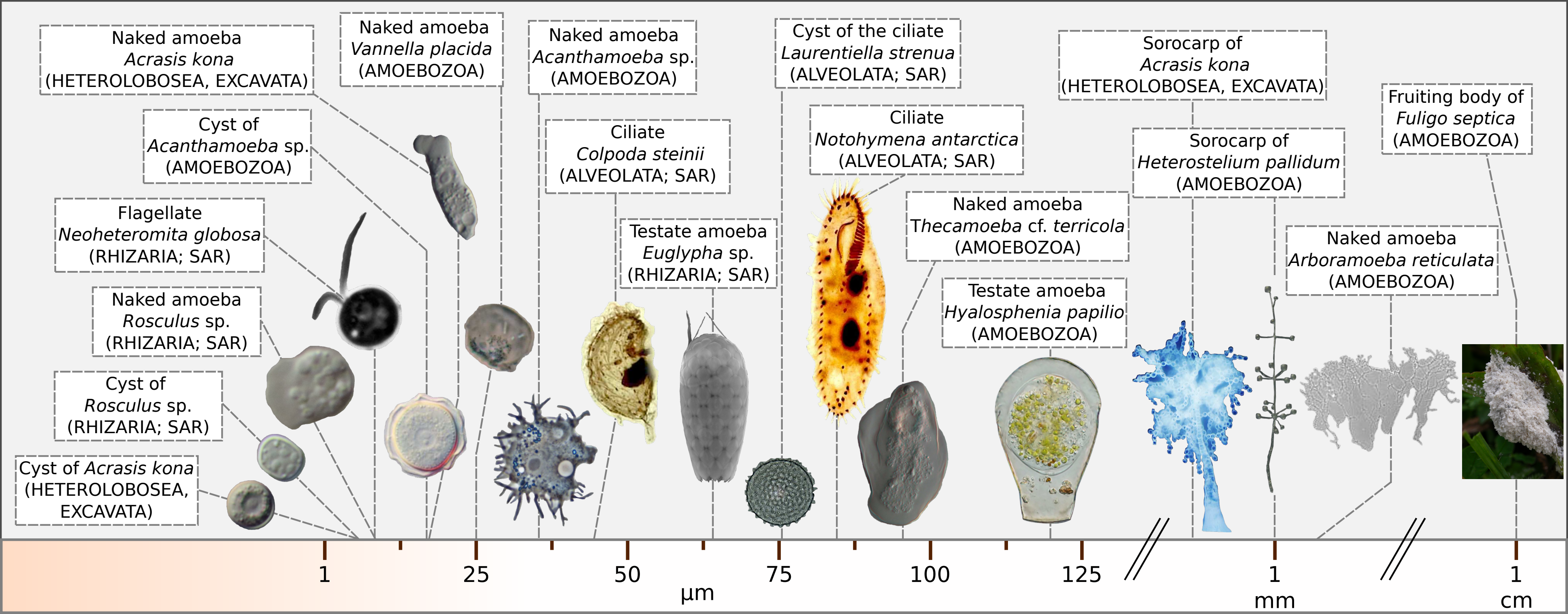
Figures

Fig. 1: Common free-living soil protists as visualized by size (lengths), morphology and phylogenetic affiliation. Note, soil protists belong to a wide range of supergroups (in brackets), whereas animals are only placed in the supergroup Opisthokonta. Furthermore, soil protists span a much wider size range as commonly assumed. With the exception of ciliates, morphogroups are not phylogenetically conserved and are placed in different eukaryotic supergroups. Most soil protists can occur in different life forms including active form (amoeba, flagellate, ciliate), but most form cysts, while some can form special reproduction structures (sorocarps and fruiting bodies).

Fig. 2: (a) Overview of studies specifically mentioning protists in the title in 5-year intervals since 1980. Soil studies represent only about a fraction of aquatic studies (separated into freshwater, marine and those that more broadly indicate aquatic) showing a strong increase in protist research in aquatic, but not soil environments; (b) Comparison of soil studies specifically mentioning protists in the title with those on other micro-sized organisms including viruses (blue filled circles), archaea (green open circles), bacteria (red diamonds), fungi (orange crosses) and nematodes (green triangles). See Supplementary Methods for details on the search.



Naked amoeba
Acrasis kona
(HETEROLOBOSEA, EXCAVATA)
Naked amoeba
Vannella placida
(AMOEBOZOA)
Naked amoeba
Acanthamoeba sp.
(AMOEBOZOA)
Cyst of the ciliate
Laurentiella strenua
(ALVEOLATA; SAR)
Sorocarp of
Acrasis kona
(HETEROLOBOSEA, EXCAVATA)
Fruiting body of
Fuligo septica
(AMOEBOZOA)
Cyst of
Acanthamoeba sp.
(AMOEBOZOA)
Ciliate
Colpoda steinii
(ALVEOLATA; SAR)
Ciliate
Notohymena antarctica
(ALVEOLATA; SAR)
Sorocarp of
Heterostelium pallidum
(AMOEBOZOA)
Flagellate
Neoheteromita globosa
(RHIZARIA; SAR)
Testate amoeba
Euglypha sp.
(RHIZARIA; SAR)
Naked amoeba
Thecamoeba cf. terricola
(AMOEBOZOA)
Naked amoeba
Arboramoeba reticulata
(AMOEBOZOA)
Naked amoeba
Rosculus sp.
(RHIZARIA; SAR)
Testate amoeba
Hyalosphenia papilio
(AMOEBOZOA)
Cyst of
Rosculus sp.
(RHIZARIA; SAR)
Cyst of Acrasis kona
(HETEROLOBOSEA,
EXCAVATA)
1
25
50
75
100
125
µm
1
mm
1
cm

A
Protist studies
marine
freshwater
aquatic
soil
number of publications
2500
2000
1500
1000
500
0
1980 - 1985
1985 - 1990
1990 - 1995
1995 - 2000
2000 - 2005
2005 - 2010
2010 - 2015
B
Soil studies
bacteria
fungi
nematodes
archaea
viruses
protist
number of publications
4000
3000
2000
1000
0
1980 - 1985
1985 - 1990
1990 - 1995
1995 - 2000
2000 - 2005
2005 - 2010
2010 - 2015

Highlights

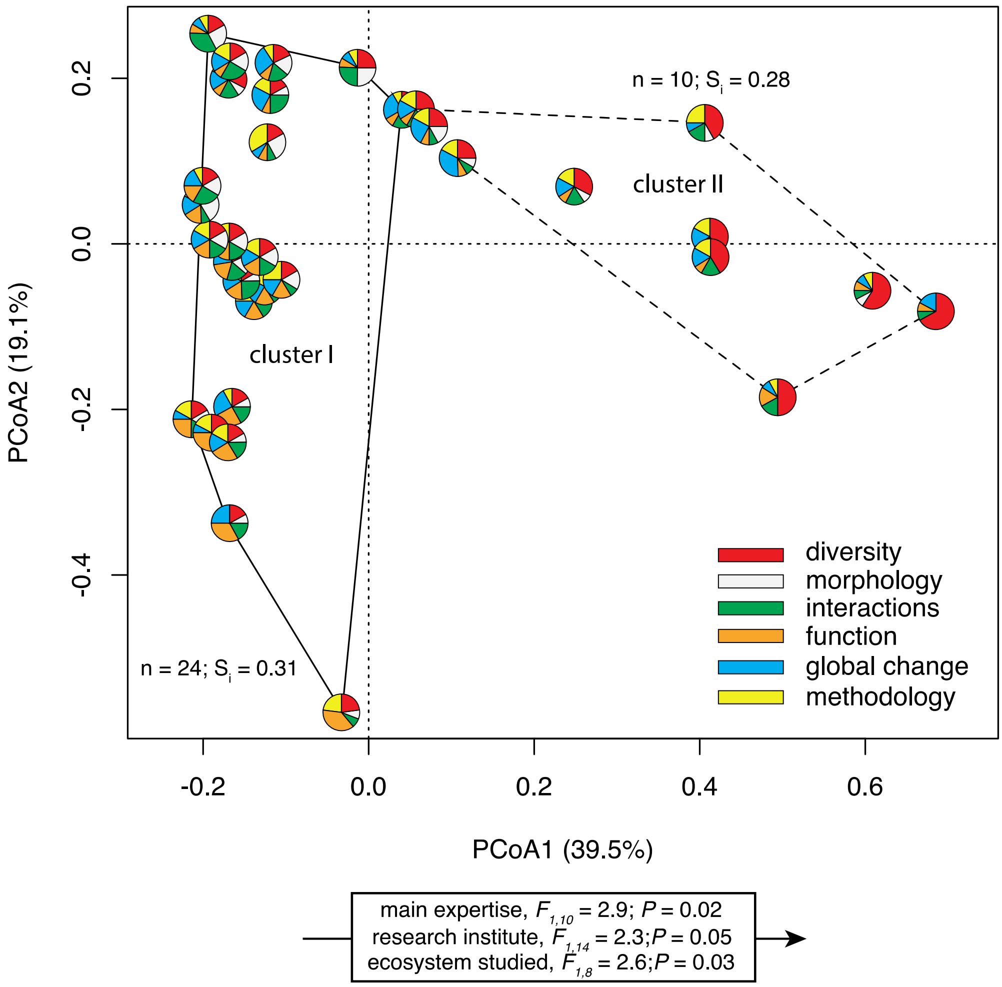
- Protists are the most diverse eukaryotes in soils
- They are key elements in the soil food web and are essential for plant functioning
- Nevertheless, protists are highly understudied compared to other microorganisms
- We here provide an overview of missing research gaps to guide future studies
- This will allow bridging protistology to general microbiology and ecology in soils

We aimed at providing a list of major questions without ranking them by numbers of votes. Despite we obtained these numbers (Supplementary Table 1), we restrained from including them into the main text as the questions were often hard to compare in terms of their scope and due to the bias included through the selection of participants – which, however, turned out to be absent or minor (see bias results below). We therefore include an overview of the analyses based on votes here.

Most votes (117) were placed into the category (ii) Diversity, Community Composition and Biogeography. (iv) Functions of Protists (103), received second most votes (99), followed by (vi) Methodology (99), (i) Morphology, Phylogeny, Taxonomy, Evolution and Physiology (81), (iii) Interactions among Protists and other Organisms (80), and (v) Global Change, Bioindicators and Applications (76). However, the highest number of votes per question was found for the categories with the least numbers of questions, i.e. (v) Global Change, Bioindicators and Applications (6.3 votes per question) and (i) Morphology, Phylogeny, Taxonomy, Evolution and Physiology (6.2 votes per question). We note that both rankings are biased, as most votes were placed in the categories where most questions were sent while the highest vote per question was affected by the necessity that researchers place at least one vote in each of the six categories.

Potential biases

Based on the votes, the fuzzy c-Means ordination showed that participants aggregated in two clusters (Supplementary Figure 1): cluster I composed of 24 participants and cluster II composed of 10 participants. The silhouette width showed that all participants well belonged to their respective clusters with an average silhouette of 0.31 and 0.28 for cluster I and II, respectively. Cluster I was composed of participants which equally voted to the proposed categories of questions, while cluster II was characterised by participants who mostly voted in the category Diversity, Community Composition and Biogeography. Main expertise, research institute and the type ecosystem studied mainly explained such grouping in the ordination space (Supplementary Figure 1). Cluster II was indeed characterised by participants from the same research area (phylogeny, taxonomy, and evolutionary), studying multiple systems and/or working in the same institution. Furthermore, variables such as experience, age, type of position and group of interest were not significantly related to the fuzzy c-Means ordination space.



Supplementary Figure 1: Principal co-ordinate ordination associated with c-Means fuzzy clustering representing the dissimilarity of votes among participants. Solid and dashed lines define the different clusters found along axis PCoA1. The average membership of the participants (sites) to each cluster can be seen with the silhouette width value (S_i). Pie charts show the proportion of votes for each question category and for each participant occurring in clusters I and II. Significant effect of explanatory variables (ANOVAs) between the PCoA axes and the individual explanatory variables are shown in boxes.

Supplementary Table 1: Questions sent for the final vote with category, total number of votes and references that (partially) addressed the respective question before.

Nr	Question	Category	Votes	References
1	How long can protists survive in an encysted form? What are the tolerances of (encysted) protists to stress and what is the importance of cysts for ecosystem resilience?	1	14	[1-6]
2	How much morphological and genetic variability exists within soil protists?	1	11	[7-15]
3	How do species that occur in both aquatic and soil systems adapt to differing demands?	1	9	[16]
4	What are the phylogenetic relations of true soil to aquatic protist taxa and how often have soils been colonized by aquatic protists and vice versa?	1	7	[8, 146-148]
5	How widespread is sex in soil protists?	1	7	[17, 18]
6	What is the real diversity and community structure of soil protists in different systems (e.g. soils, rhizosphere, (plant) endosphere)?	2	11	[26-29]
7	How similar are the diversity patterns of soil protists and other soil biota along ecological gradients, and to what extent do different environmental factors shape their respective diversity?	2	10	[30-37]
8	What abiotic environmental factors influence the distribution and community composition of protists, and how?	2	10	[30, 32, 37-48]
9	How cosmopolitan are protists and how many endemic soil protist species are there?	2	10	[49-60]
10	What are dominant groups of soil protists in terms of turnover, abundance and biomass?	2	7	[44, 62, 164, 165]
11	How do protist taxa affect the composition of the soil microbiome and what other important interactions take place?	3	14	[64-74]
12	What are the biotic interactions of soil protists with other taxonomic groups, and how are protists linked within the soil food web?	3	12	[44, 66, 75-83]
13	What is the relative contribution of nutrient cycling (i.e. the microbial loop) versus modification of the rhizosphere microbiome in protist-induced stimulation of plant growth?	3	9	[44, 82, 84-88]
14	What are the mechanisms by which individual soil protist species affect plant performance, and do those mechanisms differ between plant species?	3	8	[78, 85, 88-93]
15	What is the impact of protists on the community functioning of other soil microbes?	3	7	[74, 94-98]
16	What is the importance of soil protists in biogeochemical cycling?	4	18	[26, 99-106]
17	How much functional redundancy is there in the soil protist community?	4	12	[107-109]
18	Does increased protist diversity affect ecosystem functioning?	4	12	[26]
19	What is the comparative importance of eukaryotic microbes vs. prokaryotes in driving key soil processes?	4	9	[93]
20	Which individual functions are performed by distinct groups, and what is the entire functional diversity of	4	9	[110, 111]

	soil protists?			
21	How do changing climatic patterns affect the diversity of, community structure of and ecosystem services provided by soil protists?	5	12	[36, 112-116]
22	Which protist clades can be used as bioindicators to assess soil properties, ecosystem state, and anthropogenic impacts? How could this be implemented?	5	11	[44, 47, 82, 117-127]
23	Why are some species more sensitive to environmental change than others, why do some respond faster to environmental factors?	5	10	[128]
24	How can protists be used for nutrient mobilization and biocontrol in cropping systems?	5	8	[129]
25	What is the importance of soil protists for biodiversity conservation and ecosystem management and restoration? Should we protect particular species or habitats?	5	8	[130-133]
26	What is the most practical taxonomic unit to measure protist protist diversity?	6	20	[9, 11, 106, 134-136]
27	How can we standardize and calibrate cultivation based and molecular methods to reliably quantify soil protist abundance, diversity and activity?	6	9	[44, 137, 138]
28	How should sampling be performed to adequately evaluate soil protist diversity?	6	9	[138-140]
29	At what scales (temporal, spatial/physical, morphological, phylogenetic) should we study protists to fully understand their diversity and function in soil; which one should be prioritized?	6	8	
30	How can we infer functional traits of soil protists based on morphology or phylogenetic affiliation, and what taxonomic resolution is needed?	6	7	[26, 118, 128, 141, 142]
31	How much change in soil protists has there been over geological time?	1	7	[19-25]
32	Which taxa should be prioritized for genome and transcriptome sequencing analysis? And how should they be selected?	1	7	[143-145]
33	To what extent does horizontal gene transfer drive protist communities and ecosystem function/ecosystem services?	1	6	[149]
34	How do protists defend themselves against abiotic and biotic stressors?	1	5	[47, 150-152]
35	How often has social behaviour evolved in soil protists and do social protists dominate terrestrial ecosystems?	1	3	[153]
36	Can low Reynolds number physics be used to explain the reality of so called adaptations of soil protists?	1	2	
37	How did the association between intracellular (potentially human pathogens) bacteria and their soil protist hosts evolve?	1	2	[154, 155]
38	Could bacteria and bacterial feeding soil protists be used as a model to experimentally study genome evolution - such as horizontal gene transfer events?	1	1	[156-158]
49	Do soil protists have latitudinal, longitudinal and altitudinal diversity patterns? If so, are these patterns the same as those exhibited by macroorganisms?	2	7	[30, 37, 49, 159-163]

40	How much of the soil protist community is active in different ecosystems - what part, when, and why?	2	7	[61-63]
41	How do protist communities vary over time?	2	6	[138, 166-175]
42	How does the micro-heterogeneity of soils affect protist diversity and distribution?	2	6	[46, 108, 176-181]
43	What types of processes (i.e. ecological, historical, evolutionary) produce latitudinal, longitudinal or altitudinal diversity patterns in soil protists?	2	6	[37, 48, 182, 183]
44	How are soil protist communities structured in the soil profile?	2	5	[41, 174, 184-192]
45	How localized is the adaptation of protists to environmental conditions and environmental changes?	2	5	[193-196]
46	How much of the environmental DNA of protists in soils does not not belong to any organism actually living there?	2	4	[197]
47	What is the importance of historical factors in shaping soil protist communities in comparison to local conditions?	2	4	[35, 37, 183, 198]
48	How resilient / resistant is the active/dormant soil protist community?	2	4	[44, 45, 199, 200]
49	What is the diversity of active parasitic protists in soils and what are their hosts?	2	3	[32, 201-204]
50	What is the intraspecific diversity of soil protists?	2	3	[205-210]
51	Which soil habitats host the highest abundance and diversity of soil protists?	2	3	[175, 180, 211-217]
52	What are (unique) adaptations for protists to inhabit soils?	2	2	[195, 218]
53	How many and which soil protist taxa species have an expanding distribution, are invasive or are threatened of extinction?	2	2	[219]
54	Which are keystone taxa of soil protists in certain systems and how can we find them?	2	1	[220]
55	What is the diversity of soil protists that serves as intracellular refuges for human and animal pathogenic microorganisms?	2	1	[221]
56	How (phylogenetically) widespread is the ability of soil protists to live under anaerobic conditions?	2	0	[222]
57	What are the main parasites and pathogens of soil protists, and what impacts do they have?	3	5	[223-228]
58	How important are protist-protist interactions in structuring soil protist communities?	3	4	[99, 229]
59	Is there an especially strong connection between bacteria and soil protists as both groups have interacted for more than a billion years, and how does it compare with other microbes interacting in soils?	3	4	[230]
60	How do plant species (e.g. via exudates, volatiles etc.) affect the composition of soil protist communities?	3	4	[91, 231]
61	Is there a link between morphology and body size of soil protists and does this link to their position and role in food webs?	3	3	[72, 232]
62	Which protistan traits are most useful to predict in situ trophic interactions between bacterivorous soil protist and bacteria?	3	2	
63	Which signals/receptors are involved in food recognition of soil protists?	3	2	[233-238]
64	How does the parasitic relationship between soil protist host and parasite establish itself?	3	1	[228, 239]

65	What is known about mutualistic associations between soil protists and other organisms?	3	1	[240-242]
66	What mechanisms are used by soil protists to crack (physically) or lyse (enzymatically) their prey?	3	1	[243, 244]
67	How widespread is intra-guild predation of protists in soils (= protists feeding on other protists)?	3	1	[245-248]
68	Which chemical signals exchanged by soil protists and their prey increase stimulate protist activity resulting in e.g. increased feeding efficiency?	3	1	
69	How does addition of nutrients affect resources utilization, stoichiometry and interactions between protists and other (micro)organisms in soil?	3	1	[85, 93]
70	Are protists important for bacterial dispersion in soil, and if so which taxa are mainly responsible?	3	0	[129, 249]
71	What is the impact of protists on the process of decomposition?	4	8	[100, 250]
72	What is the abundance, diversity and functional role of the many novel and unknown soil protist clades that have been revealed by molecular environmental sampling?	4	5	[110, 251, 252]
73	What is the potential role of protists as disease suppression and biocontrol agents in soils?	4	5	[44, 82]
74	What is the relative functional importance of non-bacterivorous soil protists and how does this differ between soils/biomes/ecosystems?	4	4	[32, 253]
75	How can we extrapolate soil protist activity, diversity, and community changes to those of general microbial and higher trophic level communities, and consequently ecosystem functioning?	4	4	[93]
76	How much C is channelled through protists to higher trophic levels in soil?	4	4	[254]
77	What is the importance/function of (plant- and animal-)parasitic protists in soil?	4	4	[32, 255]
78	What are the main metabolic pathways that affect soil protist communities and ecosystem function?	4	3	[256]
79	What is the primary production input from soil protists?	4	2	[26, 241]
80	Which soil protist groups belong to the rare biosphere and are they functionally important?	4	2	[7, 201, 252, 257]
81	What is the proportion of omnivorous soil protists and are they functionally important?	4	1	
82	How does environmental filtering impact soil protist functional diversity between species (trait selection) or within species (phenotypic plasticity)?	4	1	[47]
83	What drives productivity in soil protists?	4	0	[258, 259]
84	Could soil protists be a novel mine of genetic resources with potential value for human well-being?	5	8	
85	How can we maximise the accuracy and precision of protist bioindicators?	5	6	[119, 183, 260-262]
86	How do anthropogenic activities affect soil protists?	5	6	[219, 263-268]
87	How can we model community changes including soil protists in response to global change?	5	5	[232, 269, 270]
88	Were there rapid local adaptations within soil protists that may have lead to niche changes during the Holocene, thereby obscuring palaeoreconstructions.	5	1	[128]
89	How can soil protists be used for resource production such as hydrogen?	5	1	[271]
90	How does a reduction of habitat space (e.g. due forest conversion) alter trophic level positions of indiviual	5	0	

	or the entire community of soil protists?			
91	How can we develop a universal, molecular high-throughput sequencing approach to target the entirety of soil protist diversity?	6	6	[201, 272, 273]
92	How should protist (functional) diversity be treated so as to be incorporated in food web/microbial loop models?	6	6	[138, 274]
93	How can we define standard protocols for measuring the most important functional traits for the ecology of soil protists?	6	5	[117]
94	How can we use ecological network analysis (or other such association techniques) to link protists with other soil organisms or functions?	6	5	[275-280]
95	How can eDNA and genomic experiments targeting soil protists reveal the most meaningful, functional results?	6	4	[281, 282]
96	How can we use soil protists as model organisms to study community assembly, biodiversity, trophic interactions, and eco-evolutionary processes?	6	4	[283]
97	Do transcriptome molecular genetic analyses reliably reveal the genetic basis for the ecophysiological roles of soil protists under different conditions?	6	3	[7, 64, 284]
98	How many, and which, soil protist clades are missed with current molecular methods as well as with cultivation based methods and how can we identify them?	6	2	[7, 145, 284]
99	How can we select ecologically relevant soil protist "model species" to investigate plant-fungal-bacteria interactions in specific types of soil?	6	2	[44]
100	How can we define standard protocols for using soil protists in micro-mesocosm experiments (e.g. in eco-evo studies)?	6	2	[283]
101	How can we best visualise the distribution and form of protists in soils (e.g. Micro-CT)?	6	2	
102	How reliable are identifications of soil protists based solely on morphology (no molecular barcoding) to answer (palaeo-) ecological questions and can they be optimized?	6	2	[285]
103	How consistent are results obtained in different ecological and taxonomic studies of soil protists considering identification biases, different resolutions and evolving taxonomy?	6	1	[202]
104	Which methods allow food preferences of protists to be studied in vivo?	6	1	[221, 245]
105	What is, and how can we access, the 'long-branch biosphere' (those with highly diverging sequences in barcoding regions) of soil protists?	6	1	[145]
106	Which soil protists are really unculturable and which can be cultivated using appropriate media/conditions?	6	0	
107	How can we design representative "synthetic soil protist communities" that successfully establish in soils?	6	0	

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Supplementary Table 2: Terms used to identify differences of protist research between different aquatic versus soil habitats (see Figure 1A).

	Habitat terms	*Protist terms*
Soil	Soil Soils Terrestrial	Alga Algae Algal Protist Protistan Protists Protozoa Protozoan Protozoans
Freshwater	Fresh water Freshwater Lake Pond River	
Marine	Marine Ocean Salt water Sea	
Aquatic	Aquatic Water Waters	

Supplementary Table 3: Terms used to compare research efforts for different soil organisms (see Figure 1B).

	Habitat terms
Soil	Soil Soils Terrestrial

	Organism terms
Protists	Alga Algae Algal Protist Protistan Protists Protozoa Protozoan Protozoans
Fungi	Fungal Fungi Fungus
Nematodes	Nematoda Nematode Nematodes
Bacteria	Bacteria Bacterial
Archaea	Archaea Archaeal Archea
Viruses	Bacteriophage Bacteriophages Phage Phages Viral Virus Viruses

Supplementary Table 4: Numbers of papers with the respective search terms in different time intervals (See Figure 1).

		1800-1980	1981-1985	1986-1990	1991-1995	1996-2000	2001-2005	2006-2010	2011-2015	2016
Protist terms	**Soil**	989	257	196	255	268	273	234	232	41
	Freshwater	2603	740	732	783	966	1108	1415	1482	150
	Marine	4334	1095	1023	1174	1445	1685	1950	2209	200
	Aquatic	1795	564	473	622	758	1072	1301	1344	142
	Freshwater, marine and aquatic	8732	2399	2228	2579	3169	3865	4666	5035	492
Common soil organisms	**Protists**	989	257	196	255	268	273	234	232	41
	Fungi	2686	796	749	887	1048	1358	2038	2612	321
	Nematodes	1689	434	477	530	611	684	970	979	84
	Bacteria	2202	541	554	793	1180	1559	2799	3881	491
	Archaea	0	0	0	0	12	33	123	283	27
	Viruses	514	141	184	154	118	162	239	87	14

Supplementary Table 5: Summary of the explanatory variables used to describe the participants

Variable	**What does it describe?**
Main and secondary expertise	This describes the research area of the participants
Research institute	This shows where participants are working
Age	Age of the participants
Type of position	Whether the participants are Professors, Scientists (PhD but not Professor), Post docs or PhD students
Number of publications on protists	This shows the experience of participants on the subject
Main topic	Whether protists are the main interest of the participants
Ecosystem studied	The type of ecosystem studied (e.g. forests, grasslands, wetlands, peatlands, freshwater, etc)
Morphogroup of interest	Whether the participants focused on diverse morphogroups or specifically on amoebae, ciliates, heterotrophic flagellates, naked amoebae, microeukaryotes, testate amoebae, etc